REMARKS/ARGUMENTS

Objections to the Specification

The Examiner objected to the misspelling of "Discussion" between paragraphs [0191] and [0192] of the published application, U.S. 20070054849. The Examiner also objected to the recitation of alleged hyperlinks throughout the specification. Applicants have addressed these matters in the Amendments to the Specification.

Status of the claims

Upon entry of this amendment, claims 16, 21, 25, and new claims 26-29 are pending. Claims 1-15, 17-20, and 22-24 are canceled. Claim 16 has been amended to incorporate the subject matter of claim 17, and is further supported by, *e.g.*, paragraphs [0105], [0107], and [0109] of the published application, U.S. 20070054849. Claim 21 is amended to incorporate the subject matter of claim 22, and is further supported by, *e.g.*, paragraphs [0105], [0107], and [0109]. Support for the amendment to claim 25 is found, *e.g.*, in paragraph [0107].

Support for new claims 26-29 is found throughout the specification as filed. In particular, claims 26 and 27 further define the subject matter of claims 25 and 16, respectively. Specific support for claim 28 is found, e.g., in paragraphs [0179] and [0187]. Support for claim 29 is found, *e.g.*, in paragraphs [0099] and [0107]. No new matter is added.

Rejection under 35 USC § 112, first paragraph - Enablement

Claims 15-17, 21, and 22 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. According to the Examiner, one of ordinary skill in the art would be unable to the practice the invention commensurate with the scope of the claims without an undue amount of *de novo* trial and error experimentation. The Examiner asserts (i) that the effectiveness of any given antisense polynucleotide is unpredictable, and that a skilled artisan would therefore be unable to predictably design a sequence to effectively inhibit *MGC47816*; (ii) that cancer is unpreventable; and (iii) that a skilled artisan

would have no expectation that an antisense sequence that is effective *in vitro* would be effective *in vivo*. To the extent the rejection applies to the present claims, Applicants respectfully traverse.

As set forth in MPEP § 2164.01, the test of enablement is whether one reasonably skilled in the art could make or use the claimed invention from the disclosures in the specification, coupled with information known in the art, without undue experimentation. The fact that experimentation may be complex does not necessarily make it undue, particularly if the art typically engages in such experimentation. In other words, the test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

In this case, Applicants have confirmed the ability of certain siRNA sequences, particularly those targeting SEQ ID NO: 19 of *MGC47816*, to inhibit the growth of hepatocellular cancer cells (*e.g.*, paragraphs [0179] and [0187]). However, solely in an effort to expedite prosecution, Applicants have amended the claims to not only require a specific target sequence (*e.g.*, SEQ ID NO: 19) but also to require a specific functionality (*i.e.*, disruption of the expression of the *MGC47816* gene and inhibition the function of the *MGC47816* protein). Thus, Applicants respectfully submit that the "trial and error" testing needed to identify functional siRNA oligonucleotides is no longer required. We also respectfully submit that the scope of the amended claim, and newly added claim 28, which is directed to a pharmaceutical composition comprising the claimed siRNA, clearly correlates with the scope of enablement.

Regarding the Examiner's contention that it is not possible to prevent cancer, Applicants have, solely in an effort to expedite prosecution, removed the term from the claims. In so doing, Applicants make no admissions as to the appropriateness of the rejection.

Regarding *in vivo* efficacy, Applicants draw a corollary between the enablement of a therapeutic composition and its utility and operability, which are addressed in MPEP § 2107. MPEP § 2107.01 and § 2107.03 explain that an Applicant need not demonstrate that the invention is completely safe. Furthermore, Applicants need not prove clinical efficacy to show

that a therapeutic process is operable (*i.e.*, enabled). As stated in MPEP § 2107.01, the "courts have found utility for therapeutic inventions, despite the fact that an applicant is at a very early stage in the development of a therapeutic regimen" or that a therapeutic treatment regimen is not at a stage where it is ready to be practiced on humans. *Cross v. Iizuka*, 753 F.2d 1040, 224 U.S.P.Q. 739 (Fed. Cir. 1985); *In re Brana*, 51 F.3d 1560, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995).

Moreover, MPEP § 2107.03 explains that it is not within the province of the USPTO to require proof of efficacy in animals prior to granting a patent that encompasses therapeutic methods. "Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials. There is no decisional law that requires an applicant to provide data from human clinical trials to establish utility [*i.e.*, operability & enablement] for an invention related to treatment of human disorders" (MPEP § 2107.03). The guidelines further state that "[t]he Office must confine its review of patent applications to the statutory requirements of the patent law," and that "FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws".

Thus, while the law clearly indicates that working examples are not required, it seems the Examiner is not only requiring explicit, detailed working examples but further requiring that the scope of the claimed invention be identical to that of the examples (Office Action, bottom of page 6). However, all that is required is that a "reasonable correlation" exists between the scope of the claims and the scope of enablement. In other words, if the art is such that a particular assay or model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995). Since the initial burden is on the Examiner to give reasons for lack of enablement, he must also give reasons for a conclusion of lack of correlation for an *in vitro* or *in vivo* animal model example. Importantly, a rigorous or an invariable exact correlation is not required. See *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985).

As to the Examiner's challenge to the correlation between Applicants' *in vitro* data and the expectation of positive *in vivo* results, it seems that the Examiner finds the entire field of gene therapy to be fundamentally and irredeemably unpredictable. In fact, from the context of the instant rejection, one would presume that an Applicant could never obtain protection for a claim that encompasses *in vivo* gene therapy without both conclusive human clinical trial data and extensive detail regarding administration parameters. Even then, one would be restricted to the specifics of the embodiments actually tested (*i.e.*, specific vector, administration route, disease, *etc.*).

This is in conflict with MPEP § 2107, as set forth above. Applicants need not prove clinical efficacy to show that a therapeutic process is operable (*i.e.*, enabled).

In the instant case, Applicants have conclusively demonstrated that *MGC47816* is specifically up-regulated in hepatocellular carcinoma and that small interfering polynucleotides directed to the coding sequence of *MGC47816*, particularly SEQ ID NO: 19 thereof, can disrupt the expression of the gene and suppress the growth of hepatocellular carcinoma cells (see Figures 5A and 5B). Given that the HCC cell lines tested (*e.g.*, Alexander and SNU449 cells) are routinely used as models for HCC systems *in vivo*, we respectfully submit that a reasonable correlation indeed exists between the scope of the claims and the scope of enablement and the working examples.

As evidence that the art was aware that SNU449 cells are a reliable tool for predicting the effect of test agents in animals, Applicants submit **Exhibit A,** *Li et al.* (2003) Cancer Res. 63, 3593-3597. *Li* discloses that siRNA that effectively suppressed growth of SNU449 cells *in vitro* was also effective in an *ex vivo* model using nude mice (page 3594, right column, fourth paragraph). Thus, a skilled artisan can easily understand and predict the therapeutic effect of siRNA that has been shown to inhibit SNU449 growth *in vitro*.

We further wish to remind the Examiner that the proper standard for compliance with enablement is not absolute predictability but *objective enablement*. In that vein, supporting evidence need not be conclusive but merely *convincing*. Applicants respectfully submit that the compelling data presented in the instant specification is sufficiently convincing that one of ordinary skill in the art would not doubt the feasibility of the claimed invention or its application

to higher mammals, including humans. Moreover, the *in vitro* successes documented in the Examples of the instant specification clearly outweigh any speculative allegations of unpredictability.

Contrary to the Examiner's suggestion, the "scaling up" of the disclosed procedures for application to other mammals, including humans, is considered routine experimentation well within the purview of one of ordinary skill. Thus, given the explicit disclosure in the specification of specific *in vitro* working examples, Applicants respectfully submit that one reasonably skilled in the art would be able to make and use invention without undue experimentation.

In view of the foregoing arguments and amendments to the claims, Applicants respectfully submit that the scope of the claims is commensurate with the scope of the instant specification. Accordingly, Applicants respectfully request withdrawal of the rejection under the first paragraph of 35 USC § 112 for enablement.

Rejection under 35 USC § 102(e)

Claims 21, 22, and 25 stand rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by Khvorova et al. (US 2007/0031844). According to the Examiner, Khvorova teaches an siRNA sense strand sequence, SEQ ID NO: 1564004, which comprises a nucleotide sequence of the instant SEQ ID NO: 19.

The Examiner asserts that a sense strand comprising a nucleotide sequence of SEQ ID NO: 19 can be any sense strand containing 2 consecutive nucleotides of SEQ ID NO: 19, in distinction from a sense strand comprising the nucleotide sequence of SEQ ID NO: 19, which must contain all of SEQ ID NO:19. While we respectfully disagree with the Examiner's interpretation of "a" vs. "the", we nevertheless have amended the claims to recite "the nucleotide sequence of SEQ ID NO:19." Claim 25 has been amended to define the length of the siRNA as 19 to 25 nucleotides, and claim 26 has been added to recite an siRNA consisting of the nucleotide sequence of SEQ ID NO: 19.

We also note that the Khvorova application, filed November 13, 2003, claims the benefit of two provisional applications, as follows:

U.S. Provisional Application Ser. No. 60/426,137, filed Nov. 14, 2002, and
U.S. Provisional Application Ser. No. 60/502,050, filed Sep. 10, 2003.

From a review of the respective disclosures, it appears that SEQ ID NO: 1564004 was not added until the non-provisional filing (*i.e.*, November 13, 2003). In that Applicants' priority date is September 23, 2003, it appears that Applicants' disclosure of SEQ ID NO: 19 predates Khvorova's disclosure of SEQ ID NO: 1564004. Accordingly, the Examiner's suggestion that the Khvorova application is prior art under 35 U.S.C. § 102(e) that anticipates Applicants' claim to a sense strand sequence comprising a nucleotide sequence of the instant SEQ ID NO: 19 is in error.

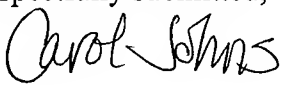
In view of the foregoing comments, Applicants respectfully request withdrawal of the rejection under 35 USC § 102(e).

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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[CANCER RESEARCH 63, 3593–3597, July 1, 2003]

Use of RNA Interference to Target Cyclin E-overexpressing Hepatocellular Carcinoma[1]

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ABSTRACT

RNA interference is the process by which double-stranded RNA directs sequence-specific degradation of mRNA. It has recently been shown that RNA interference can be triggered by 21-nucleotide duplexes of small interfering RNAs (siRNAs) in both cultured mammalian cells and adult mice. We hypothesize that siRNA can be used to specifically target oncogene overexpression in a therapeutic manner. Here, we show that overexpression of the oncogene cyclin E can be suppressed by up to 90% in hepatocellular carcinoma (HCC) cell lines by siRNA targeted on the coding region of cyclin E. We also find that depletion of cyclin E in this manner promotes apoptosis of HCC cells and blocks cell proliferation. Finally, we show that the siRNA oligos inhibits HCC tumor growth in nude mice. Thus, this study demonstrates the therapeutic potential of siRNA on the treatment of HCC by targeting overexpressed oncogenes such as cyclin E. Our results also indicate that cyclin E, which is overexpressed in 70% of HCCs, may serve as a novel therapeutic target.

INTRODUCTION

RNAi[3] is the process whereby dsRNA results in the rapid destruction of mRNA containing the identical sequence as the dsRNA. The mediators of RNAi are 21- and 22-nt siRNAs generated by RNase III cleavage from longer dsRNA (1, 2). Delivery of dsRNA has been shown to knock down the expression of specific proteins in insect cell lines. However, in most mammalian cells, it has not been possible to exert potent and specific effects by applying dsRNA (>38 bp) because long dsRNA also induces a nonspecific inhibitory response resulting from the IFN pathway (3). Recently, it has been shown that delivery of 21-nt siRNA specifically suppressed expression of endogenous and heterologous genes in different mammalian culture cells and mice (4–7). We sought to use siRNA targeting a common oncogene to determine whether this technique can be used to specifically inhibit oncogene overexpression and whether this inhibition results in antitumor effects.

HCC is the third leading cause of cancer death worldwide, with an estimated 564,000 new cases and almost as many deaths in 2000 (8, 9). Currently, there is no effective therapy for the vast majority of HCC patients. Therefore, the understanding of the molecular mechanisms involved in HCC formation and progression become critical to developing more effective treatments for HCC. Genetic analyses have revealed that one of the commonly altered genes in HCC is the cell cycle regulator cyclin E (10). Cyclin E is believed to control G_1-S-phase progression. By associating with cyclin-dependent kinase Cdk2 and activating its kinase activity shortly before entry of cells into the S phase (11, 12), its expression near the G_1-S-phase transition is thought to be critical for the initiation of DNA replication and duplication of the centrosomes. The timely appearance and disappearance of cyclin E is crucial: excessive activity of the cyclin E-Cdk2 complex drives cells to copy their DNA prematurely, resulting in genome instability (13) and carcinogenesis (14). In fact, clinical studies have indicated that cyclin E plays an important role in HCC formation and progression. Overexpression of cyclin E was found in ~70% of HCC patients, which correlated with the poor prognosis of those patients (15). We choose to study the applicability of RNAi as therapy against cyclin E overexpression in HCC by *in vitro* and *in vivo* experiments.

MATERIALS AND METHODS

Cell Culture. HCC cell lines, Hep3B, HepG2 and SNU449 were obtained from American Type Culture Collection (Manassas, VA) and HuH7 was a generous gift of Dr. Patricia Marion (Stanford University, Stanford, CA). Hep3B and HepG2 are cyclin E-overexpressed lines, and HuH7 is a cyclin E-nonoverexpressed line (16). The cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum. All cells were maintained in a humidified 37°C incubator with 5% CO_2.

Transfection with siRNA Oligos. The siRNA oligos were synthesized by Dharmacon Research, Inc. The siRNA oligos corresponded to nts 361–382 of the human cyclin E coding region (GenBank accession no. XM_049430). The indicated HCC cells (1×10^5/well) were transfected with siRNA oligos (0.3 μg/well) in 6-well plates using Oligofectamine reagent (Invitrogen) following the manufacturer's protocol.

Cell Cycle Analysis. Standard fluorescence-activated cell sorter analysis was used to determine apoptosis of the cells or the distribution of cells in cell cycle. Briefly, the cells were transfected with cyclin E siRNA or other agents. Adherent cells were then collected by trypsinization and combined with cells floating in the medium. The apoptotic cells were assessed by flow cytometric detection of sub-G_1 DNA content after being stained with propidium iodide.

TUNEL Assay. Apoptotic cells were confirmed using the *In Situ* Cell Death Detection kit from Roche (Mannheim, Germany), following the manufacture's instruction. The apoptotic cells (red staining) were counted under a microscope. The apoptosis index was defined by the percentage of red cells among the total cells of each sample. Six fields with >100 cells in each were randomly counted for each sample.

BrdU Incorporation. Thirty h after transfection of siRNA, the cells were split into 4-well chamber slides and incubated with culture medium containing BrdU for 4 h or 20 h. BrdU staining were performed using Zymed BrdU labeling kit (Zymed, San Francisco, CA) following the manufacturer's protocol.

Western Blotting. Forty-four h after transfection, cells were lysed, as indicated, into mammalian cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) with 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethyl sulfonylflouride, 2 mg/ml aprotinin, and 2 mg/ml leupeptin]. After centrifugation at 4°C (14,000 rpm, 15 min), lysates (20 μg) were analyzed by immunoblotting. Anticyclin E polyclonal antibody (C-19) was from Santa Cruz Biotechnology, and antiactin antibody (Ab-1) was from Oncogene Research (Boston, MA). Images were quantitated by NIH-Image software.

Colony Formation Assay in Soft Agarose. The standard colony formation assay was used (17). Briefly, HCC cell line Hep3B was transfected with siRNA oligos targeting cyclin E or LacZ. Two days after the transfection, the cells (1×10^3 cells/well) were plated in 24-well plates in culture medium containing 0.35% agarose overlying a 0.7% agarose bottom layer and cultured at 37°C with 5% CO_2. Five weeks later, the top layer of the culture was stained

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[3] The abbreviations used are: RNAi, RNA interference; dsRNA, double-stranded RNA; nt, nucleotide; HCC, hepatocellular carcinoma; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; BrdU, bromodeoxyuridine.

Exhibit A

(5 pages total)

with *p*-iodonitrotetrazolium (1 mg/ml). Colonies >100 μm in diameter were counted.

***Ex Vivo* Tumor Inhibition.** Hep3B cells were transfected with or without siRNA oligos. Forty-four h after transfection, 2×10^6 cells were s.c. injected into nude mice (*nu/nu*, 8–9 weeks of age; Harlan Sprague Dawley, Madison, WI). The volume of the resulting tumor was measured weekly. The difference in tumor volume between treated groups was compared for statistic significance using the unpaired, two-tailed *t* test.

RESULTS

Suppression of Cyclin E Overexpression in HCC by RNAi. Cyclin E is commonly overexpressed in HCC and correlated with poor prognosis of those patients (10). To address if cyclin E could serve as a therapeutic target for this cancer, we used siRNA oligos to deplete cyclin E expression in HCC cells. We designed one pair of siRNA targeting the coding region of cyclin E (Fig. 1*A*). The siRNA oligos were transfected into three cyclin E overexpressing HCC cell lines, Hep3B, HepG2, and SNU449. Forty-four h after transfection, the protein lysates were harvested and analyzed by anticyclin E Western blotting. We found that cyclin E expression levels were suppressed by up to 90% in all three cell lines (Fig. 1*B*, *Lanes 2*, *8*, and *11*). This suppression was also detected as early as 24 h after transfection. These data indicated that the siRNA could effectively suppress cyclin E overexpression. The inhibitory effect of the cyclin E siRNA was shown to be specific because a control oligo-targeting LacZ gene had no effect on cyclin E expression levels. In addition, siRNA oligos did not cause a nonspecific down-regulation of gene expression, as demonstrated by the actin control (Fig. 1*B*). The effects of the siRNA were durable as we observed the suppression even 5 days after transfection (data not shown).

Induction of Apoptosis and Inhibition of DNA Synthesis in HCC Cells by RNAi Depletion of Cyclin E. Three days after the transfection of cyclin E siRNA, we observed that cells with cyclin E overexpression shrank, rounded up, and detached from plates, suggesting apoptosis had occurred. In contrast, the lacZ siRNA control group remained attached on the dishes and showed normal morphology. To determine whether depletion of cyclin E promotes tumor cell death, flow cytometry was performed after transfection of siRNA oligos into Hep3B, HepG2, and SNU449. The cells were analyzed at different time points (72 and 96 h) after transfection and significant sub-G_1 (apoptotic) populations were observed at 96 h (Fig. 2*A*). We found that 16% of Hep3B cells underwent apoptosis after transfection of siRNA oligos *versus* 1% in the control group. The apoptotic population was even higher when HepG2 and SNU449 cells were tested (44 and 31%, respectively; Fig. 2*A*). As a control, we transfected LacZ siRNA into these three overexpressing cells, and no significant apoptosis was observed (data not shown). To test if apoptosis can be triggered in cells without cyclin E overexpression, we also transfected cyclin E siRNA into HuH7, a cyclin E-nonoverexpressing HCC cell line. In contrast to cyclin E overexpressed cells, we did not observe significant apoptosis in HuH7 at 72 or 96 h after transfection (Fig. 2*B*), although its cyclin E protein was effectively suppressed by cyclin E siRNA (Fig. 1*B*, *Lane 5*). We also confirmed the apoptosis of Hep3B by TUNEL assay (Fig. 2*B*). These data together suggested that depletion of cyclin E specifically triggered apoptosis in cyclin E-overexpressing cells.

To determine whether cyclin E overexpression is required for G_1-S-phase transition and replication in cancer cells, we examined the rate of DNA synthesis of siRNA-treated Hep3B cells by BrdU incorporation assay. Thirty h after transfection, the cells were incubated with BrdU for 20 h followed by the BrdU staining. Fewer (51%) BrdU-stained cells (brown color) were observed in the cyclin E siRNA-treated group compared with those in mock treatment (66%) or the LacZ siRNA control group (65%), indicating that depletion of cyclin E-suppressed Hep3B DNA synthesis (Fig. 2, *C* and *D*). We also performed BrdU incorporation assay in both HepG2 and SNU449 cells and found similar results (Fig. 2, *C* and *D*). To address if depletion of cyclin E affects the cell distribution in the cell cycle, we performed flow cytometry 32 h after transfection. Our results showed a decreased cell population in S phase after cyclin E siRNA treatment in all three cell lines compared with control, as shown in Fig. 2*E*. Taken together, our results indicate that the cyclin E siRNA exhibited a specific inhibitory effect on cyclin E-overexpressing HCC through promotion of apoptosis as well as inhibition of DNA synthesis.

Cyclin E siRNA Inhibits Cancer Cell Growth and Suppresses Tumor Formation in Nude Mice. Next, we sought to determine whether cyclin E siRNA could serve as a therapeutic agent against HCC tumor formation in nude mice. We first tested the effect of cyclin E siRNA on the growth of Hep3B, HepG2, and SNU449 in cell culture. As shown in Fig. 3*A*, after transfection, cyclin E siRNA significantly inhibited cell growth of all three cell lines as compared with LacZ siRNA control or mock treatment. The inhibitory effect on Hep3B cell growth was confirmed by soft agar assay as shown in Fig. 3*B*. In contrast, we did not observe dramatic growth inhibitory effect on HuH7 cells (data not shown).

The antitumor activity of siRNA in Hep3B cells was additionally assessed using an *ex vivo* assay. Hep3B cells were first transfected in Petri dishes with or without cyclin E siRNA. The transfected cells were then injected into nude mice, and the growth of tumors was measured weekly. As shown in Fig. 4, cyclin E siRNA significantly suppressed tumor growth in mice as compared with control, indicating that targeting cyclin E by siRNA can exert a strong antitumor effect *in vivo* on cyclin E-overxpressing HCC.

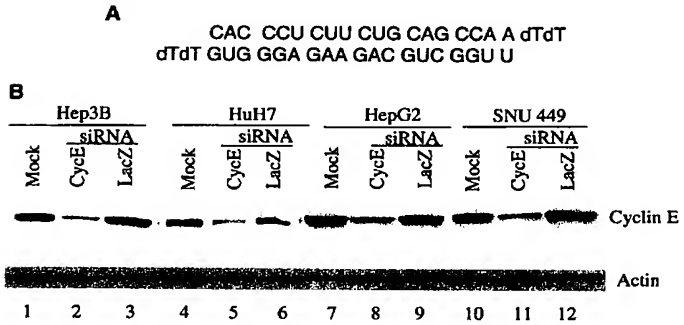
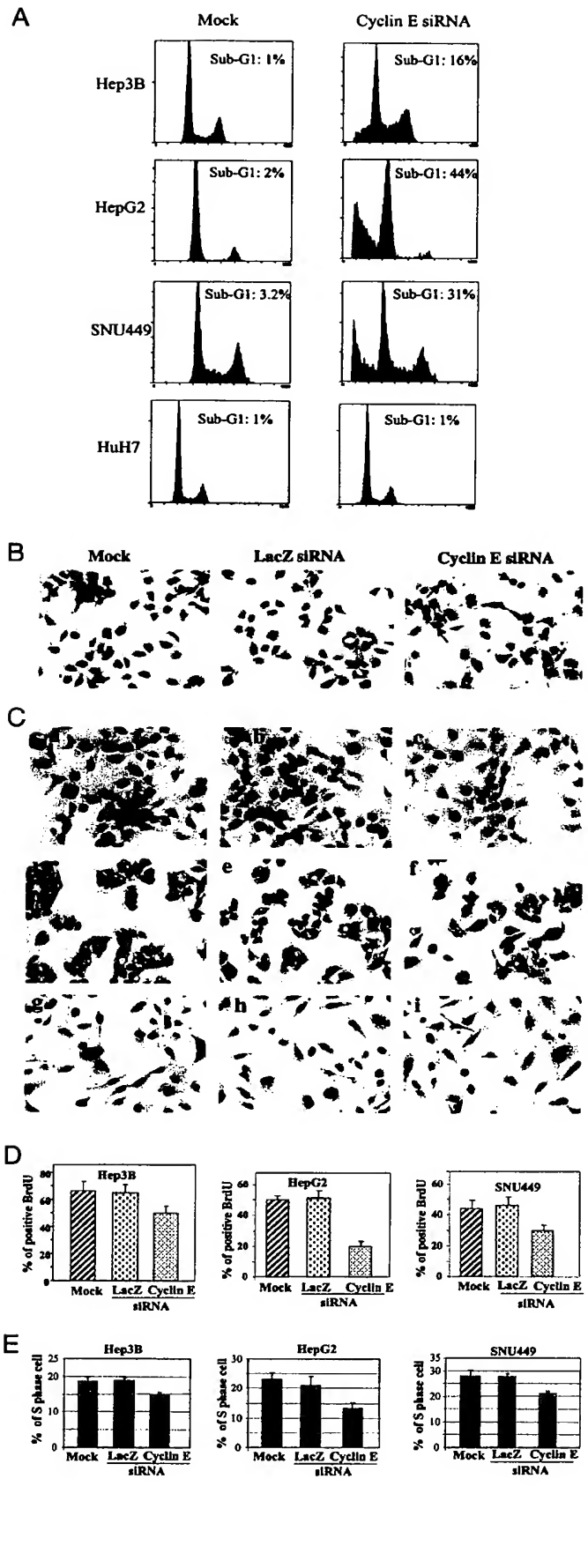


Fig. 1. Depletion of cyclin E overexpression by siRNA oligos in HCC cells. *A*, the sequence of 21-nt siRNA duplex that were used to target on cyclin E. *B*, indicated cells were transfected with siRNA oligos targeting on either cyclin E or LacZ (control). Cells were harvested at 28 or 44 h after transfection. The protein lysates were subjected to anticyclin E and antiactin Western blot. Images were quantitated by NIH-Image software.

DISCUSSION

Oncogene overexpression has been implicated in the development and progression of a variety of human cancers and, therefore, provides a potential target for cancer gene therapy (10). For years, research has focused on effective tools to specifically down-regulate oncogene overexpression such as antisense oligonucleotide strategy. However, there has been only limited success because of the lack of specificity and potency for this method (18). For example, screening of >20 oligomers is usually required before identifying one antisense that functions effectively, and the dose required for inhibiting gene expression is often not much different from doses that lead to nonselective toxicity.

The recent progress of RNAi techniques has demonstrated the



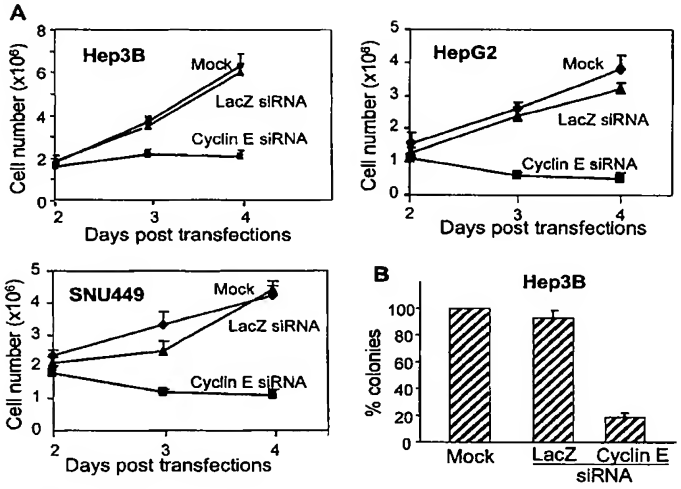


Fig. 3. Inhibition of cell growth by cyclin E siRNA *in vitro*. *A*, growth curves of Hep3B, HepG2, and SNU449 cells in response to siRNA. The viable cells were counted at the indicated time points. The data shown here represent the averages from four independent experiments. *B*, suppression of colony formation in soft agar. Hep3B cells were transfected with siRNA targeting on cyclin E or LacZ and then seeded in 0.35% agarose containing DMEM with 10% fetal bovine serum. The cells without any oligo transfection (mock) were used as controls. The colony numbers were counted 5 weeks later. The numbers of colonies for the treated cells were then standardized against the control cells (set at 100%). The data were the averages from two independent triplicate experiments; *error bars*, SD.

potential to overcome those limitations. The selection of the targeting sequences of RNAi is less restricted, so the success rates of producing effective duplexes are higher (18). In addition, siRNA is dsRNA, which is more resistant to nuclease degradation as compared with antisense oligos and, therefore, have longer therapeutic effects than the antisense approaches. A recent study directly compared these two techniques and found that siRNA appeared to be quantitatively more efficient with more durable in cell culture (19). When tested in mice, only siRNA but not antisense oligos exhibited the inhibitory effects. This sequence specificity and its effective inhibitory effects have recently been successfully applied to suppress cancer cell growth induced by point mutation-activated Ras (20) or by BCR/ABL fusion gene (21). Our studies were designed to test whether siRNA can also been applied to selectively target cancer cells with overexpression of oncogenes, which is a more common cause of oncogene activation compared with point mutations and chromosome translocations.

Our results demonstrate that siRNA can effectively down-regulate oncogene overexpression with great specificity. As shown, the siRNA

Fig. 2. Induced apoptosis and reduced cell proliferation by depletion of cyclin E. *A*, down-regulation of cyclin E promoted apoptosis of Hep3B, HepG2, and SNU499 but not HuH7 cells. Ninety-six h after the siRNA transfection, the indicated adherent cells were collected by trypsinization and combined with cells floating in the medium. The apoptotic cells were then determined with flow cytometry. Three individual experiments were performed, and the cell distribution in cell cycle was determined by standard fluorescence-activated cell sorter analysis. The cell population in sub-G_1 was shown. The *X* and *Y* axes represented DNA content and the cell number, respectively. *B*, TUNEL assay to detect apoptotic cells induced by siRNA. Hep3B cells were transfected with cyclin E siRNA and LacZ siRNA, respectively. Seventy-two h after transfection, the cells were analyzed for apoptosis using TUNEL assay. *Red nuclei* staining indicated apoptosis. *C*, decreased replication rate induced by cyclin E siRNA. Thirty h after transfection of siRNA, BrdU was added into the medium, and the cells were incubated for another 20 h and subjected to BrdU incorporation assay. The cells with *brown color* in nuclei were BrdU-positive cells. *a–c:* mock, lacZ siRNA, and cyclin E siRNA treatment in Hep3B; *d–f:* mock, lacZ siRNA, and cyclin E siRNA treatment in HepG2; *g–i:* mock, lacZ siRNA ,and cyclin E siRNA treatment in SNU449. *D*, schematically showing of BrdU-positive cells from *C*. *E*, decreased S-phase population after transfection of cyclin E siRNA for 32 h. The S-phase population in the indicated cells was determined by flow cytometry.

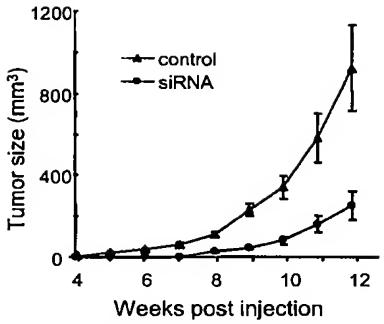


Fig. 4. *Ex vivo* assay for tumor suppression effect of cyclin E siRNA. Hep3B cells were transfected with cyclin E or the control siRNA in culture plates. Forty-eight h later, viable cells (2×10^6) were injected s.c. into the right and left flanks of five mice in each group. Tumor formation was scored weekly. *Error bars:* SD.

oligos could successfully deplete up to 90% of cyclin E in Hep3B, HepG2, and SNU449, three cell lines that each express at least 10-fold higher cyclin E than that in normal cells, indicating the potency of RNAi as a new strategy for cancer therapy. Also, the blockage of proliferation and induction of apoptosis in cultured cells and the tumor suppression effect in nude mice additionally support the effectiveness of this treatment. The induced apoptosis was only observed in cyclin E-overexpressing cells (Hep3B, HepG2, and SNU449) but not in cyclin E-nonoverexpressing cells (HuH7), and this specificity should increase the therapeutic index of RNAi-based therapies.

After cells were treated with cyclin E siRNA for 30 or 44 h, we observed a significant decrease of DNA replication in both cyclin E-overexpressed cells and the noncyclin E-overexpressed cell HuH7 (data not shown). The decreased replication rate as determined by BrdU incorporation is consistent with our flow cytometry results showing a decrease of the S-phase population 32 h after transfection. Cyclin E has been known to play an important role at G_1-S transition, as well as DNA replication (11, 12). Therefore, the slowdown of DNA synthesis may have resulted from both the blockage of S-phase entry and DNA replication. Interestingly, in addition to the inhibition of DNA synthesis, depletion of cyclin E for 72 or 96 h triggered apoptosis in all three cyclin E-overexpressing cell lines that we tested. It is unclear how cyclin E depletion triggered apoptosis in cyclin E-overexpressing cells at this point. However, our flow cytometry data suggest that there might be different mechanisms to trigger apoptosis in different cell lines depending on the unique genetic context in the individual lines. For example, in HepG2 cells, depletion of cyclin E-induced apoptosis occurred after a dramatic increase of the G_1 population and a decrease of S and G_2 cells (preliminary data not shown), suggesting that the failure of S-phase entry may somehow trigger cell death in these cells. In contrast, Hep3B cells transfected with cyclin E siRNA underwent apoptosis after an increase of G_2-M phase cells. It suggests that after cyclin E is depleted, the cells with incomplete DNA replication, instead of being blocked at intra-S phase, may enter G_2-M phases. Thus, apoptosis could be triggered as a consequence of the cells trying to undergo mitosis in the presence of unreplicated DNA.

Interestingly, when apoptosis was induced (72 or 96 h) in Hep3B cells by depletion of cyclin E, we observed an increase of S-phase cells, which is not seen in the earlier (32 h) time point before apoptosis was triggered. We suspect that the increase of S-phase accompanying apoptosis may be an artifact from the incomplete DNA fragmentation of G_2-M cells. Although all cells that complete apoptosis possess a unified DNA content as sub-G_1, the cells in the middle of this process would likely contain a variable amount of DNA. For example, the G_2-M cells with partially fragmented chromosomes would have DNA content between 2N and 4N, which is indistinguishable from the S-phase cells on flow cytometry profiles. In fact, for the same flow cytometry experiment, if we excluded the cells floating in the medium (apoptotic population), we then observed a decrease of S phase in the cyclin E-siRNA-treated group as compared with the control at 96 h (data not shown). Thus, this suggests that this increase of S phase at 96 h might result from the incomplete DNA fragmentation of G_2-M apoptotic cells.

In addition to demonstrating the use of RNAi in cancer gene therapy, our results also indicate that cyclin E might serve as a novel therapeutic target for HCCs with cyclin E overexpression. Cyclin E has been suggested as an attractive target for molecular therapeutics both because it is overexpressed in a significant fraction of human tumors, including HCC (10), and because this overexpression is implicated in tumor formation (14). It has been postulated that interference with cyclin E expression or function could inhibit the neoplastic growth of a wide variety of cancers (10). However, to date, there has been no report to experimentally support this theory. The hurdles may have resulted from the lack of approaches to effectively deplete cyclin E in cancer cells. Here, we showed that when overexpressed cyclin E was depleted in HCC, it resulted in multiple antitumor effects in cancer cells such as blockage of DNA replication and induction of apoptosis *in vitro* and reduction of tumor growth *in vivo*. These results suggest that cyclin E overexpression may be essential for maintaining cell proliferation, as well as cell survival in cyclin E-overexpressing HCC. This is the first study, to our knowledge, showing that targeting cyclin E overexpression is a potential effective approach to treating cyclin E-overexpressing HCC.

In this study, we directly transfected synthetic siRNA oligos, which allowed us to evaluate their therapeutic effect on cancer cells. In fact, siRNAs can also be expressed from plasmid or viral vectors using the RNA polymerase III promoter (20, 22), and the expression may be combined with a tumor-specific promoter or an inducible system (23) such that siRNA can specifically target oncogenes in cancer cells without affecting normal cells. Future studies will investigate whether cyclin E overexpression can be efficiently depleted by siRNA expressed from a DNA-based expression vector.

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